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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: David E. Fisher

Application No.: 09/229,283 Group No.: 1642
Filed: 1/13/1999 Examiner: UNGAR, Susan
For: USE OF MICROPHTHALMIA FOR DIAGNOSIS, PROGNOSIS AND/OR
TREATMENT OF MELANOMA

THIRD DECLARATION OF DAVID E. FISHER UNDER 37 C.F.R. 1.132

I, David E. Fisher, am the inventor of the above-described invention. I declare as follows:

1. I am aware that the Examiner has raised a question concerning whether there is an adequate written description of "an antibody that selectively binds human Mi" in the specification.
2. I explained in my previous declarations that the present application teaches a method for diagnosing melanoma that involves contacting a biological sample containing malignant cells with a probe that recognizes microphthalmia (First Declaration of David E. Fisher, paragraph 6). I also explained that, at the time the application was filed, we were abbreviating the name as Mi, but since that time, the protein has been referred to as microphthalmia associated transcription factor and typically abbreviated "MITF" or "MITF" (Ibid).
3. I discovered that the expression of Mi in a malignant cell is indicative of melanoma. I explained in my First Declaration that one preferred method for determining the expression of Mi was using an antibody that selectively recognizes the expression of Mi (See First Declaration, paragraph 15).
4. I further explained that it was not necessary that that antibody distinguished between the different isoforms of Mi, but merely that the antibody selectively binds to Mi (First Declaration, paragraph 15).
5. The skilled artisan knows that an antibody that selectively binds to a protein means that the antibody would not cross-react with a wide range of proteins. In other words, it must have

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the ability to be able to sufficiently discriminate between multiple proteins to accomplish the desired result.

6. In the specification, we exemplify preparing an antibody that selectively binds to human Mi, namely, the monoclonal antibody D5. We point out that you can use the amino terminal Taq-Sac fragment of human Mi cDNA and use this to generate an antibody that will selectively bind to human Mi.

7. At the time this application was filed, the skilled artisan was well-aware of how to prepare antibodies. The sequence of human Mi was known. Indeed, a discussion of Mi and Mi mutants and analogues is set out at pages 5 and 6 of the specification. Methods for preparing such antibody are provided in the specification at pages 13 - 15.

8. Homologous regions with related proteins were known.

9. It is known that one can generate antibodies using fragments that are as small as six amino acids. Methods for enhancing the ability of a particular fragment to generate an amino acid that will bind to that fragment are known.

10. Consequently, the skilled artisan, based on what I taught and demonstrated in view of the state of the art, knew what was being described.

11. Moreover, it was well known at the time of filing that using a phage display system, antibodies could be made completely *in vitro*, bypassing the immunization step. Such a method would rely only on screening to identify an antibody with the desired property, i.e., one that will selectively bind Mi. (See *Huogenboom*, "Designing and optimizing library selection strategies for generation of high affinity antibodies" (TIB TECH, February 15: 62 - 70)(1997)).

12. The skilled artisan knows that antibodies, unlike many other types of proteins, and certainly nucleic acids are typically not defined by an amino acid sequence or a nucleotide sequence, but rather by their ability to bind a particular protein. This is because of how well known and standard these procedures are in the art.

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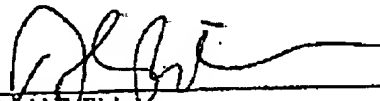
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13. In terms of the type of guidance that is necessary here, one only needs an antibody that binds Mi. I previously indicated, it was not necessary for the antibody to distinguish between different isoforms of Mi.

14. It is not necessary for this antibody to bind to some specific binding site on the protein because one is not looking to block some activity as one might with certain therapeutics. Rather, the goal is to identify whether or not Mi is present.

15. It is my understanding that the Examiner has argued that because antibodies are screened and tested to ensure they have the desired activity, there is not a written description of such an antibody. I disagree. Selection of a desired type and affinity of produced antibodies by screening is part of any method of making antibodies. For example, Hoogenboom shows a routine method of making antibodies in Figure 1 at page 64. In this method, one first constructs a library and then enriches the antigen-specific antibodies using affinity selection. Since the early '80's, when people were producing monoclonal antibodies, the screening of hybridomas to select those that expressed an antibody with a desired characteristic was always considered a necessary and routine part of the manufacture of such an antibody. This is how the field looks at and describes antibodies.

16. I hereby declare that all statements made herein of my own knowledge are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.



David E. Fisher

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Designing and optimizing library selection strategies for generating high-affinity antibodies

Hennie R. Hoogenboom

Since its invention at the beginning of the 1990s, antibody phage display has revolutionized the generation of monoclonal antibodies and their engineering. It is now possible to create antibodies binding to any chosen target antigen without the use of laboratory animals or hybridomas, in a system that completely bypasses the immune system. Making antibodies from single-pot phage libraries, and improving their affinity up to the picomolar range if necessary, has never appeared easier. In this review, a variety of phage library-based strategies for the isolation of high-affinity antibodies are presented.

The biotechnological generation of high-affinity monoclonal antibodies has traditionally involved the production of hybridomas from spleen cells of immunized animals. Now the use of phage antibodies offers a new route for the generation of antibodies, including antibodies of human origin, which cannot be

easily isolated by conventional hybridoma technology. Over the last decade, protein engineering methods to generate more human-like antibodies have been developed¹ (chimerization and humanization of rodent antibodies) and, more recently, transgenic mice expressing human antibodies were proposed (reviewed in Ref. 2). In all cases, antibodies are created or engineered with their specificity and affinity shaped by the immune system. With phage display, antibodies can be made completely *in vitro*, by-passing the immune system and the immunization procedure, and allowing in

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in vivo tailoring of the affinity and specificity of the antibody. As for other methods, the first commercially significant application of phage antibodies is the generation of fully human monoclonal antibodies for medical use. In fact, the use of phage display is set to gradually replace hybridoma technology in all of its present applications, in both research and in the diagnostic industry.

Antibody phage display³ is accomplished by fusing the coding sequence of the antibody variable (V) regions to the amino terminus of the phage minor coat protein, pIII. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody being presented on the phage surface, while its genetic material resides within the phage particle. This linkage between antibody genotype and phenotype allows the enrichment of antigen-specific phage antibodies, using immobilized or labelled antigen. Phage that display a relevant antibody will be retained on a surface coated with antigen, while non-adherent phages will be washed away. Bound phages can be recovered from the surface, re-infected into bacteria and re-grown for further enrichment and, eventually, for binding analysis. The success of antibody phage display hinges on the combination of this display and enrichment method, with the creation of large combinatorial repertoires of phage antibodies. Each of these antibodies has a different antigen combining site, generated by a PCR-based amplification of antibody V-genes (reviewed in Ref. 4). An antigen-driven enrichment procedure can be used to isolate even the rarest phage antibody. The complete procedure is illustrated in Fig. 1. This basic technique has been applied to construct very large and highly diverse combinatorial repertoires of antibody fragments, from which antigen-specific antibodies to any chosen antigen can be selected⁵. This review will focus on the applications, advantages and disadvantages of these large repertoires.

Choosing the right antibody library

The choice of which type of phage antibody repertoire to use depends very much on the application and the required affinity of the antibody. Ideally, antibodies to any chosen antigen are selected from universal, antigen-unbiased libraries. Two types of such 'single-pot' libraries have been described, namely the naive and synthetic antibody libraries. Indeed, it has been demonstrated that these can be used as a source of antibodies against any chosen antigen. The alternative is to develop and use antigen-biased phage antibody repertoires, using V-genes from immune sources. Figure 2 shows a decision tree to guide the choice of the repertoire (points 1-3), and the selection strategy (4-6), depending on a number of parameters. Figure 3 shows a comparison of the performance of different repertoires, which is discussed in detail below.

Antibodies from naive V-gene libraries

The primary immune response involves a large array of IgM antibodies that recognize a variety of antigens.

This array can be cloned as a 'naive' repertoire of rearranged genes, by harvesting the V-genes from the IgM mRNA of B cells of unimmunized human donors⁶, isolated from peripheral blood lymphocytes (PBLs), bone marrow, spleen cells or from animal sources⁷. The V-genes are amplified from the cDNA using oligonucleotides binding to the Framework (FR) 1 and 4 encoding regions of the V-gene, and heavy and light chains assembled randomly to encode single-chain Fv (scFv) or Fab antibodies. These cassettes are cloned directly into an appropriate phage or plasmid vector for expression and display. The first of such 'single-pot' repertoires⁸ was made from the PBLs of two healthy human volunteers and contained 3×10^7 clones, from which antibodies to over 25 different antigens were isolated. These included antibodies to self antigens and cell-surface molecules^{9,10}. The average affinity of these antibodies was in the 10^6 – 10^7 M⁻¹ range, which is similar to the affinity of antibodies seen in the naive primary immune response. Recently, much larger scFv repertoires have been made by 'brute force' cloning¹¹, with 10^9 – 10^{10} independent antibody clones, and from these libraries higher-affinity antibodies (K_d around 10^9 M⁻¹) have been selected. Even larger-sized naive Fab repertoires may be made by using the *in vivo* recombination system based on the *Cre/loxP* recombination system⁵. In this method, individually cloned light and heavy chain repertoires are recombined using appropriately positioned *loxP* recombination sites by P1 bacteriophage Cre recombinase, yielding Fab repertoires that in size are limited by the amount of bacteria grown, typically 10^{11} – 10^{13} members.

The major advantages of using very large naive single-pot repertoires are: (1) one library can be used for all antigens; (2) human antibodies can be isolated; (3) antibodies to self, non-immunogenic or toxic substances can be generated; (4) antibody generation takes 2–4 rounds of selection (approx. two weeks); and (5) when very large repertoires are available, high-affinity antibodies are isolated directly.

The disadvantages are: (1) the lower affinity of the antibodies when smaller-sized repertoires are used; (2) the time and effort it takes to construct these repertoires, in particular when using the *Cre/loxP* recombination system⁵; and (3) the largely unknown and uncontrollable contents of these naive libraries. In particular, the potentially limited diversity present in the IgM repertoire, its bias due to unequal expression of the different V-gene families, and the unknown immune history of the B cell donor, influence the contents and quality of the library. Therefore, an alternative method, based on the controlled addition of defined and chosen V-gene building blocks to antibody repertoires, has been developed: the synthetic antibody library.

Antibodies from synthetic V-gene libraries

The wealth of data on the *in vivo* generation of antibodies, via a complex process of DNA rearrangement, and the many antibody structures that have been

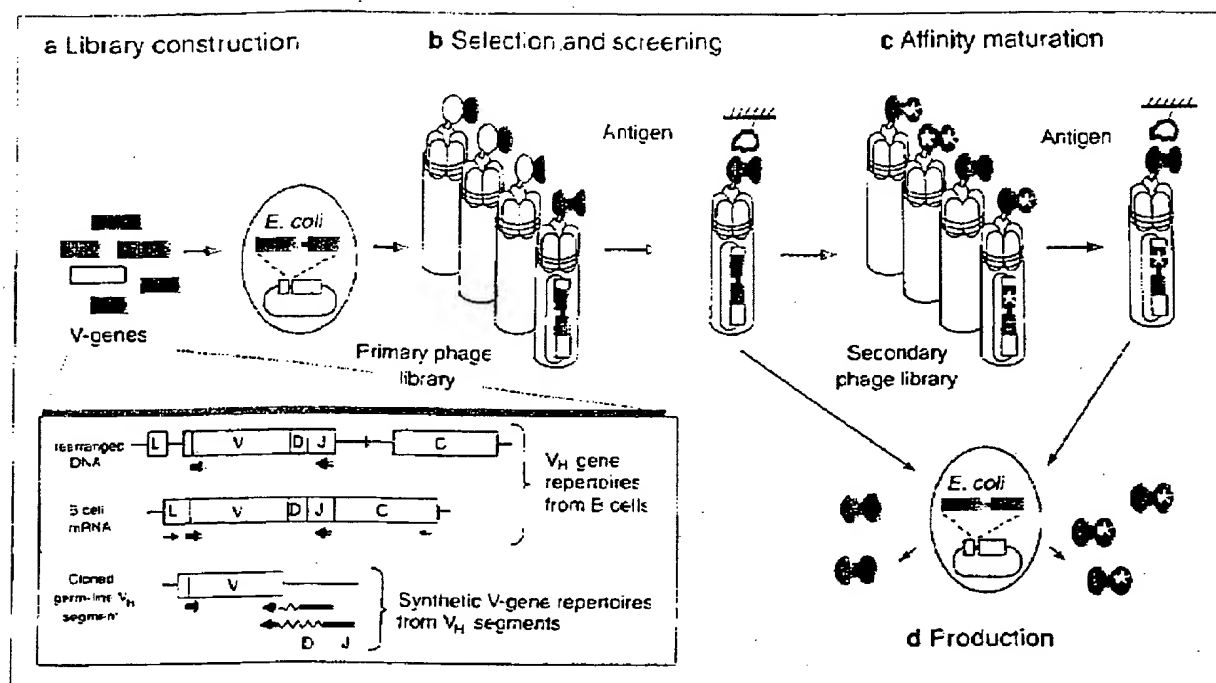


Figure 1

Overview of the phage display method. (a) Library construction: V-genes (grey and black boxes) are cloned in phage(mic) vectors for display on phage. (b) From these primary libraries, antigen-specific antibodies are enriched using affinity selection. (c) If necessary, the selected antibodies are affinity matured, by diversifying their sequence, yielding a secondary library from which the highest affinity clones are enriched. (d) Finally, soluble antibody fragments may be produced by expression in bacteria. The inset highlights the location of oligonucleotides for cloning of V_H segments, used for amplifying the rearranged genes from DNA or mRNA (for naive or immune libraries), or to provide an artificial CDR3 (as an example for synthetic libraries). L, leader; V, variable; D, diversity; J, joining; C, constant domain segments. Modified from Ref. 4.

solved have shown clearly that most of an antibody's binding strength resides in the six complementarity determining regions (CDRs) that shape the antigen combining site. This knowledge has paved the way for the creation of antibodies entirely outside their natural host. To construct a synthetic antibody library, V-genes are assembled by introducing a predetermined level of randomization of CDRs (or possibly also bordering FR-regions) into germ-line V-gene segments¹⁰, or rearranged V-genes¹¹. One of the main advantages over naive repertoires is that in such synthetic repertoires the contents, local variability and overall diversity of the library may be controlled and defined.

The areas of synthetic diversity are mostly chosen to correspond to areas of greatest natural sequence diversity of the primary antibody repertoire. From structural studies it has become apparent that five of the six CDR regions (all but the CDR3 of the heavy chain) have limited structural variation, and frequently follow a certain canonical fold¹². The greatest structural diversity is found in the loop most central to the antigen combining site, the CDR3 of the heavy chain. This loop is highly diverse in composition and in length owing to the *in vivo* recombination procedure.

The antibody CDR regions may be partially or completely randomized using oligonucleotide-

directed mutagenesis or PCR-based methods. Several synthetic or semi-synthetic repertoires have been made in which, in particular, the heavy-chain CDR3 loop was targeted. In the first synthetic antibody library, constructed in Cambridge, UK, a set of 49 human V_H-segments was assembled via PCR, with a short CDR3 region (encoding either five or eight amino acids) and a J-region, and cloned for display as a scFv with a human lambda light-chain¹⁰. From this repertoire, many antibodies to haptens, and one against a protein antigen, were isolated, delivering the first proof of concept for this synthetic antibody approach. Subsequently, the CDR3 regions were enlarged to match better the natural length diversity of this loop¹³, and eventually 47 light-chain segments with partially randomized CDR3 were added⁵. In the final repertoire a set of nearly 100 cloned human V-gene segments was used, covering over 95% of the segments used in the natural human repertoire, and therefore resembling the human primary antibody library in its diversity⁵. In other synthetic antibody repertoires, one rearranged V-gene pan was provided with a one-length randomized CDR3 region in the heavy chain¹⁴, or all three CDR-loops in one antibody V-gene were diversified¹⁵. Many different synthetic library concepts appear to work in creating antibodies of intermediate affinity

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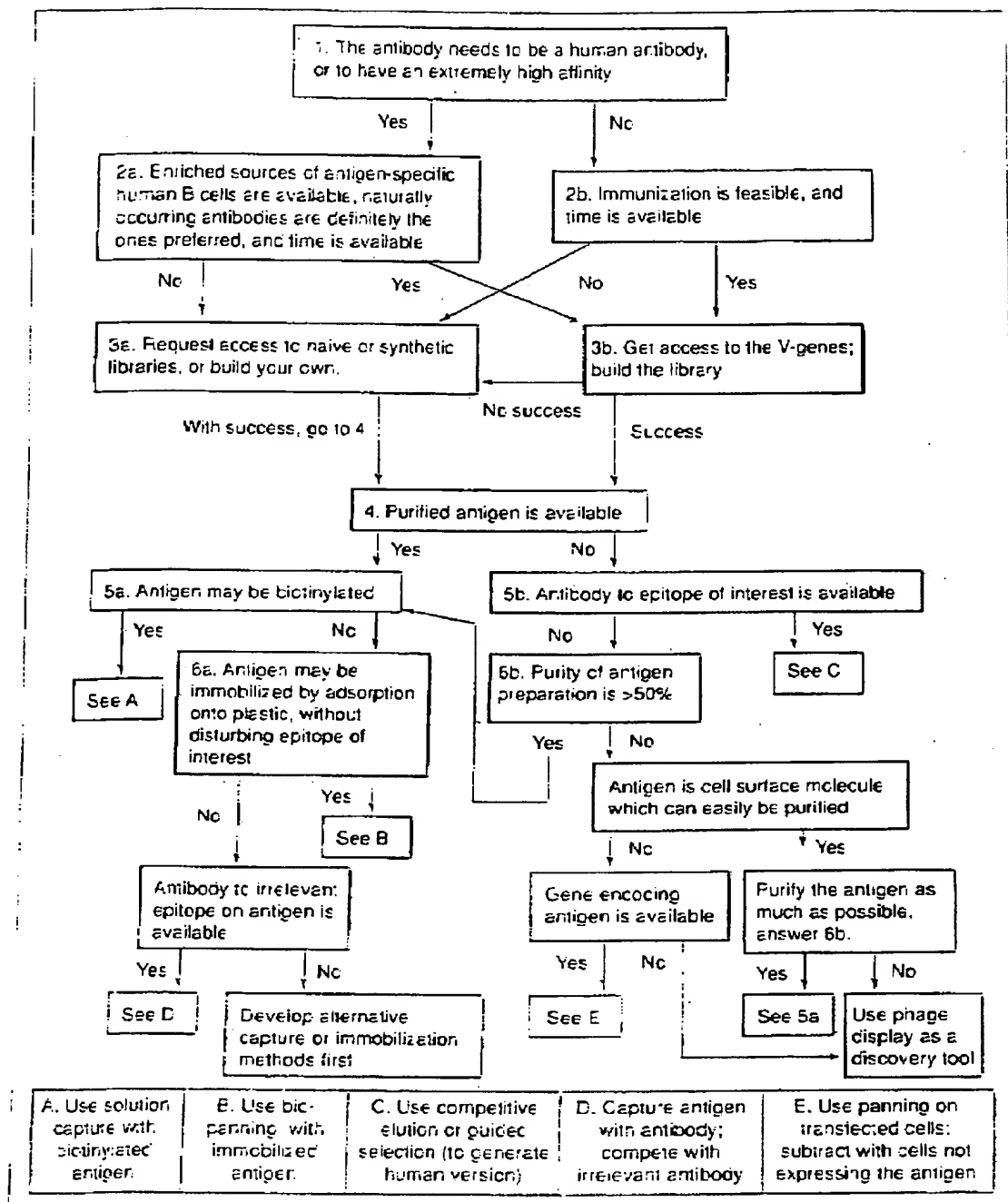


Figure 2

Flow chart to decide which type of antibody library to make or use (1-3), and which selection method to apply (4-5).

(10^6 – 10^8 M⁻¹). However, in all cases the cloning of a sufficient number of variants will become limiting. For example, when using mutagenic oligonucleotides to randomize six codons (with, at each triplet, the first two base positions A, G, C or T, and the last G or C) one billion combinations arise. Limitations in trans-

formation efficiency limit the size of the antibody library that can be made in practice to 10^9 – 10^{10} variants and, therefore, the total accessible diversity. A combination of the dual antibody cloning strategy¹² and a smart cloning system, based on *in vivo* Cre/loxP site-specific recombination, allows the construction of very

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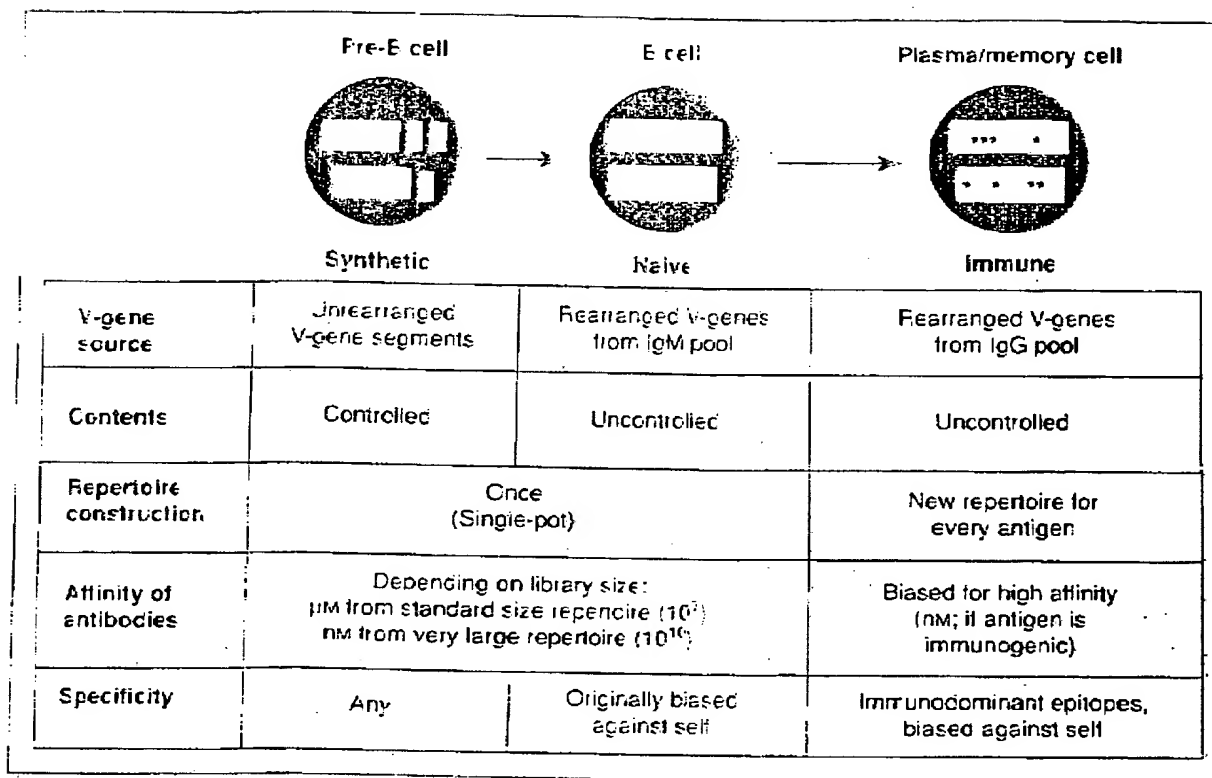


Figure 3
Comparison between synthetic, naïve and immune libraries.

large repertoires⁵, with 10^{10-12} members. By using larger repertoires, the chances of finding a higher-affinity antibody are increased. Indeed, from a repertoire with 6×10^{10} clones, Griffiths and colleagues isolated synthetic Fab fragments with high affinity (K_d 10^8 – 10^9 M⁻¹).

Artificial versus natural

Synthetic antibody repertoires containing genuine human germ-line sequences^{5,10,13} deliver antibodies that may be considered more 'natural' than those from naïve IgM-mRNA-derived repertoires, which are frequently found to be somatically mutated¹⁴. On the issue of immunogenicity, the randomized CDR3 may appear synthetic and thus foreign; however, the choice for relatively short (4–12 residue) CDR3 loops favours the lowest possible immunogenicity, without excluding fully (or with 'natural' human antibodies) anti-idiotypic responses.

One of the main advantages over naïve repertoire is that in synthetic repertoires the contents, local variability and overall diversity of the library may be controlled and defined. For example, in addition to the CDR3, 'primary' diversity may also be introduced in the subset of CDR1-2 residues that are hypervariable owing to the natural somatic hypermutation mechanism¹⁵, rather than in all CDR residues¹⁶. A particular example is the provision of the CDR3 region,

which may be performed for germ-line segments individually, allowing choice of particular segments, and avoiding the frequent cross-overs noted when PCR-amplifying V-genes in batches²³. After selection, CDR or 'Vernier' zone FR-regions may be varied at will, in parallel or sequentially. This may facilitate systematic manipulation by, for example, using standardized methods to perform affinity maturation by sequential CDR-diversification (see also below). Although the overall impression now may be that slightly better antibodies are generated from naïve⁹ than from synthetic repertoires⁵, the advantages in using synthetic antibody libraries will eventually reduce primary naïve libraries to useful but transient sources of V-genes.

Antibodies from immune V-gene libraries

For immune libraries, V-genes are derived from the IgG mRNA of B cells of an immune source, e.g. immunized animals or in some instances human immune B cells. An immune phage antibody library repertoire will be enriched in antigen-specific antibodies, some of which will also have been affinity matured by the immune system^{16,17}. This method gives access to more, and sometimes better, antibodies than when working with hybridomas. For example, from an immune murine phage antibody library, Chester and colleagues identified an anti-CEA antibody with an affinity substantially higher than ever obtained with

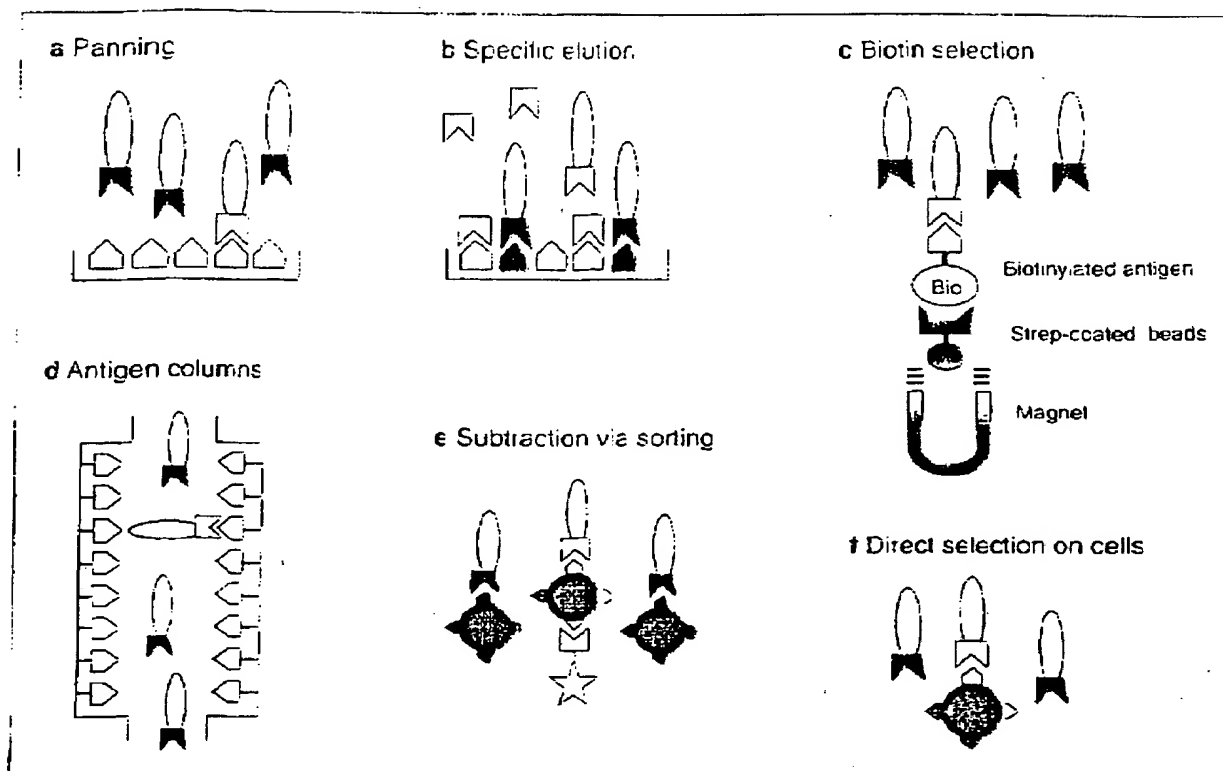


Figure 4

Most frequently used selection methods. Phages displaying antigen-specific antibodies (white) are usually separated from non-binding phages (black) using the following procedures. Antigen may be adsorbed onto Petri dishes or plastic tubes for panning (a), or immobilized on Sepharose and loaded onto columns for affinity selection (d). The antigen concentration used during selection may be monitored carefully by using antigen labeled with biotin. After incubation of phages with the biotinylated antigen, phages bound to the antigen-biotin complex are retrieved with streptavidin-coated paramagnetic beads and a magnet (c). When using impure antigen preparations or targeting a specific epitope, the antigen itself or other reagents that bind the antigen may be used to elute off epitope-specific antibodies or cover irrelevant epitopes (b). For cell surface antigens, phages may be directly selected on cells (f), or antigen-displaying cells may be sorted by virtue of a marker unique for these cells, using fluorescence or magnetic activated cell sorting (FACS or MACS, respectively) (e). Combinations and alterations between these methods may be made. After washing, the bound phages can be eluted non-specifically, with high or low pH buffers, by changing the ionic strength or by direct incubation with bacteria. A protease recognition site may be encoded between the antibody fragment and the pIII protein, allowing specific protease cleavage (see Ref. 4).

the traditional technology¹⁹. Disadvantages of this route include the time required to immunize animals, the unpredictability of the immune response to the antigen of interest, the lack of immune response to some (for self antigens, or toxic molecules), and the restriction in generating human antibodies. In addition, a major drawback of this method is that for every new antigen a new phage antibody library has to be created, which adds 1-3 months to the procedure. Commercially available kits, such as RPAS from Pharmacia Biotech for the creation of scFv repertoires from mouse or rat spleen cells, will make access to this route easier for laboratories starting this work. However, the creation of such repertoires remains a technically non-trivial procedure and, therefore, for most applications only medium-sized repertoires will be made. The use of very large naive or synthetic libraries, when accessible, may well yield antibodies with similar affinities much faster. For example, the

affinities of the best antibodies directed to the hapten 2-phenyloxazol-5-one, selected from an immune repertoire¹⁹, were of the same range as those isolated from a very large synthetic repertoire⁵. Thus, the availability of pre-made large collections of non-immune repertoires has superseded the use of immune repertoires. It should be carefully considered if immunization and the creation of animal-derived antibody libraries is required, and also whether such experiments can be justified from an ethical point of view.

Immune repertoires for specialized users

There may be a few occasions where this route will still be useful, e.g. for the analysis of the humoral immune response in man. Naturally occurring high-affinity human antibodies, for example, present after viral infection or in patients with autoimmune diseases or cancer, may be cloned by phage display with caution. The scrambling of V_H and V_L genes in the process of

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the combinatorial library construction makes it difficult to discriminate 'original' V_H - V_L combinations from *de novo* formed pairs. The likelihood of retrieving original pairs is very low and, if necessary, 'in-cell PCR', in which V_H and V_L genes are assembled within the cell¹⁹, may be used (although this method has only been demonstrated for mixed hybridomas). It has also been observed that the fine specificity may be shifted by mispairing the V-domains. Antibodies from immune random combinatorial libraries may identify the antigen or epitope(s) involved in the humoral immune response, and it may not always be strictly necessary to obtain original pairs.

In a second application of immune libraries, the immune response itself is used to remove irrelevant antibodies, thus improving the quality of the V-genes before cloning. Animals can be made tolerant for certain irrelevant antigens, after which the relevant antigen as a mixture with irrelevant antigens is used for immunization. This combination of *in vivo* and *in vitro* depletion and enrichment (tolerization and immunization *in vitro*, and depletion of phage antibodies before selection on antigen *in vitro*) should be extremely powerful, as exemplified by the isolation of human antitumour cell antibodies from phage repertoires of cancer patients immunized with autologous tumour cells²⁰.

Designing selection procedure:

Phage antibody libraries are enriched for antigen-binding clones by subjecting the phages to repetitive rounds of selection that include binding, washing and elution steps, reinfection into bacteria and growth to re-express the antibody on the phage surface. Figure 4 shows the most frequently used *in vitro* selection methods, which are routinely used when purified antigen is available; Fig. 2 addresses which of the many methods should be used. Selections on impure antigens are significantly more difficult, owing to the problem of enriching phage antibodies specific for non-relevant antigens. Depletion and/or subtraction methods, cell sorting or selection by alternating between different sources of antigen may be used. Furthermore, the use of immune libraries with a higher frequency of specific antibodies may prove beneficial²⁰. Using these fine-tuned methods, antibodies have been made to antigens previously thought of as difficult, such as to MHC-peptide complexes²¹ and human blood group antigens²².

Selection from phage antibody libraries provides a new tool for the isolation of novel self-antigens, such as tumour- or disease-associated antigens. The *de novo* combined V-domain pairs in both naive and synthetic antibody libraries (but particularly the latter) are not shaped by the constraints of the immune system, and avoid library bias by *in vivo* tolerance mechanisms. Therefore, antibodies to unique self-epitopes can be isolated, provided powerful cell panning, sorting or subtraction methods are available. To date, this application has been used for probing lymphocyte²³ and tumour cell surfaces²⁴, yielding a number of promi-

ing but as yet uncloned cell-type specific antigens. The lack of reactivity of the selected phage antibodies in immunostaining²⁵, their low affinity²⁶, or the low abundance of antigen complicates their full characterization. The isolation of 'specific' binders *per se* does not prove that these subtractive selection methods function; by direct panning on a live colorectal carcinoma cell line we recently generated a large panel of anti-tumour scFv antibodies (Zijlema *et al.*, unpublished). The exquisite epithelial cell origin of the antigen was determined by positive immunohistochemistry on colon tumours derived from cancer patients. Systematic studies to compare the various enrichment methods are required to understand and appropriately design the selection of phage antibodies on cell surfaces. Lastly, *in vivo* selections, in which the phage repertoires are injected directly into animals, may reveal phage antibodies specific for certain organ-specific antigens, as demonstrated for peptide-phages²⁷.

Designing screening assays

After the selection procedure, a fast and robust screening assay for binding is applied, ranging from a simple ELISA with coated antigen, bioassays for screening for direct neutralization upon binding, to whole cell ELISAs, FACS or immunocyto- or histochemistry. Phagemid vectors that serve a dual purpose, mediating the monovalent display of scFv or Fab fragments for selection, and allowing production of soluble antibody fragments tagged with signals for purification and detection, are widely used¹⁵. If an alternative antibody format (e.g. Fab, whole antibody) is required for the screening assay, fast V-gene recloning methods should be used²⁸. Alternatively, the direct display and selection of other formats of antibodies such as bispecific antibodies²⁹ may be envisaged.

Human antibodies via guided selection

Even when no antigen is available or the antigen is not known, it is possible to generate antigen-specific human antibodies. We have recently applied the technique of 'guided selection'³⁰ to derive a human anti-CD30 antibody, without having antigen available (Klimke *et al.*, unpublished). In this experiment, selections were carried out by panning a Hodgkin's cell line carrying the CD30 antigen on its surface, avoiding cloning, expression and purification of the antigen. Guided selection has been applied previously to generate anti-hapten and several anti-protein antibodies using standard phage selection on purified antigen. In this chain-shuffling technique, a murine antibody serves to guide the selection of the human V-domains onto the antigen³¹. In the first step, one of the murine V-domains is replaced with a repertoire of human V-domains, the rodent-human chimaeras displayed on phage and antigen-binding pairs enriched from the library. In a second step, the remaining murine sequence is replaced, resulting in a complete human equivalent of the original antibody. The selected human CDR-regions may interact with the same epitope in a very different manner from the original antibody,

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yielding similar²⁷ or worse affinities²⁸, but always antibodies targeting the same region on the antigen.

Improving affinities

By selection from very large repertoires (with $>10^9$ antibody variants), antibodies with (sub)nanomolar affinity may be obtained directly²⁹, making affinity maturation necessary in some specialized cases only. The affinity of antibodies selected from the more frequently used medium-sized naive, synthetic or immune phage libraries, however, is typically sufficient for use as research reagents, but often rather suboptimal for therapeutic applications, i.e. tumour targeting and virus neutralization. Affinity maturation may be bypassed by the construction of multivalent molecules, as reviewed in Ref. 29; however, there will be occasions in which *in vivo* affinity maturation of the selected antibodies is required. *In vivo* antibodies are affinity matured in a stepwise fashion, by gradually incorporating mutations that each cause a small incremental improvement of the affinity. Mutations may affect affinity indirectly by influencing the positioning of side chains contacting the antigen, by providing new contact residues (particularly when they are located in or near the centre of the antigen combining site) or by replacing 'repulsive' or low-affinity contact residues with contact residues with more favourable energetics. The *in vivo* affinity maturation process essentially involves three steps: (1) introduction of diversity in the antibody V-genes, creating a 'secondary' library (Fig. 1); (2) selection of the higher-affinity from the low-affinity variants; and (3) screening to allow discrimination of antibody variants with differences in affinity or kinetics of binding. The selection may be chosen to favour kinetic parameters³⁰ such as off-rate or affinity; this hinges on the use of limited and decreasing amounts of antigen, and on performing the selections in solution rather than by avidity-prone panning. For example, antibodies of the highest affinity can be preferentially selected by using the antigen concentration at or below the desired dissociation constant³⁰.

The areas that are best targeted for mutagenesis will differ for each individual antibody. Diversity may be introduced more-or-less randomly in the V-genes, as with error-prone PCR, by using mutator strains, by chain shuffling or by DNA shuffling. Chain shuffling of V-genes has been used successfully to obtain a 300-fold increase in the affinity of an anti-hapten antibody, originally isolated from a naive human scFv library³¹. In this experiment, first the light chain and then the heavy chain (without CDR3) were replaced by a repertoire of human partner domains somatically mutated *in vivo*, and from these repertoires the best variants were selected on antigen. A similar approach was applied to antibodies binding to protein antigens, with a more modest sixfold affinity improvement for an anti-c-erbB2 antibody³² but no improvement for gp120 binding Fabs (Ref. 33).

Alternatively, the antibody's CDR regions may be targeted, using oligonucleotide-directed mutagenesis or spiked oligonucleotides and PCR. With over 100

CDR residues constituting the antigen combining site, a choice will have to be made as to where to start. If the structure of the antibody-antigen complex is known, residues that contact the antigen or may influence other residues contacting the antigen could be targeted. Such residues may also be determined experimentally, by chain shuffling³⁴, alanine-scanning of the CDR regions³⁵, parsimonious mutagenesis³⁶, or modelling. Residues involved in maintaining the main chain conformation of the CDR should be conserved; residues that modulate affinity may be randomized, ideally 4–6 residues at a time to allow efficient sampling of the sequence space. Sequential targeting is preferred, as additive effects of mutants obtained by targeting CDRs in parallel and combining mutations frequently prove unpredictable³⁷. Using 'CDR-walking', Yang and colleagues³⁷ created independent libraries with mutations in one of four CDR regions, and combined the selected variants in one clone. This strategy yielded a 420-fold increase in affinity for the best anti-gp120 Fab.

Can the immune system teach us how to design mutagenesis strategies? *In vivo*, the diversity introduced by somatic hypermutation in the maturation process is mainly located at the periphery of the antigen combining site³⁸, suggesting that we should target the first and second CDR regions for *in vitro* affinity maturation. However, the best examples of affinity maturation *in vitro*, yielding in some cases picomolar affinity antibodies^{34,35,37}, highlight the importance of targeting the centre, i.e. the CDR3 of the heavy chain. The powerful *in vivo* maturation procedures described may allow specific introduction and selection of mutations by iteration, in a way not available to, but also not required by, the immune system.

Future prospects

The above mentioned picomolar-affinity human antibodies to viral antigen HIV gp120 and tumour antigen c-erbB-2 are probably the first two examples of antibodies with an affinity and exquisite fine-specificity not previously accessible. We can finally envisage clinical trials with human antibodies that are better than any antibody made before, even better than the ones made by nature.

Although originally intended for the production of fully human antibodies, phage-based methods are creating opportunities for engineering antibody affinity and specificity not previously envisaged. Without doubt, most novel features in antibody engineering will be based on the synthetic antibody concept. Downstream processing and biotechnological production may be facilitated by choosing only those V-gene segments that are expected to be expressed to high levels in the heterologous host. The V-gene building blocks may be modified or selected to incorporate a ligand binding site for purification, a reactive site for introducing probes within the antigen combining site, or CDR-loops with different canonical forms or derived from other species. In addition, the development of 'designer' libraries is envisaged, in which the

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building blocks have a propensity for binding to a certain type of antigen by virtue of choice of the CDR-side chains, or provide structurally similar antigen combining sites such as flat surfaces, pockets or canyons.

Affinity maturation strategies need to be developed that allow maturation of selected antibodies in batch mode rather than on an individual basis. Most likely, a mixture of systematic cloning and re-selection methods, or of libraries with carefully pre-mutated building blocks matching the initial design of the diversity of the primary synthetic library, will be used. Eventually, both the selection from the primary library as well as the affinity maturation event may take place in a purpose-built automated machine, possibly the ultimate goal in the biotechnological generation of tailor-made antibodies.

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